

Award Number: DAMD17-00-1-0473

TITLE: Melatonin, Aging and Breast Cancer

PRINCIPAL INVESTIGATOR: Steven M. Hill, Ph.D.

CONTRACTING ORGANIZATION: Tulane University School of Medicine
New Orleans, Louisiana 70112-2699

REPORT DATE: June 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20030930 029

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2003	3. REPORT TYPE AND DATES COVERED Final (1 Jun 00 - 31 May 03)
4. TITLE AND SUBTITLE Melatonin, Aging and Breast Cancer Pathway		5. FUNDING NUMBERS DAMD17-00-1-0473
6. AUTHOR(S) Steven M. Hill, Ph.D.		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tulane University School of Medicine New Orleans, Louisiana 70112-2699 E-Mail: kkozar@tulane.edu		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES		
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The pineal gland, via its hormone melatonin, inhibits the proliferation of both human and animal models of breast cancer. As humans age there is the onset of disrupted sleep leading to a significant suppression in the nocturnal levels of melatonin after age 60. We have hypothesized that the decline in pineal melatonin production, with the onset of old age, is a key factor in the age-related increase in breast cancer. Using the Buffalo rat model, we have begun to characterize the melatonin rhythm in young, middle aged and old female rats. Our studies demonstrate that the nocturnal rise in both serum and pineal melatonin is significantly blunted in old rats compared to middle aged and young rats, and is blunted in middle aged rats compared to young rats. As well, uterine MT1 melatonin receptor levels are greatly diminished in old female rats (by 80%) compared to young female rats. Finally, in our studies tissue-isolated transplanted mammary tumors grew significantly faster and were less melatonin-responsive in old rats as compared to middle and young aged rats. In addition, tumors grown in old rats showed decreased expression of ER α and MT1 melatonin receptor, but greatly enhanced expression of the growth factor TGF α .		
14. SUBJECT TERMS		15. NUMBER OF PAGES 16
		16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
		20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusions.....	12
References.....	13
Appendices.....	13

INTRODUCTION:

An increasing percentage of elderly women, particularly in industrialized countries, are developing breast cancer. Furthermore, it is expected that breast cancer incidence will continue to increase with increased age. A number of hypotheses have been proposed to explain this dramatic increase in breast cancer incidence in the later stages of life, including total dose of carcinogen exposure. However, an alternative hypothesis is that aging results in changes in the internal milieu of the organism, such as metabolic, endocrine and immunologic shifts, providing increasingly favorable conditions for tumor induction, promotion and progression. The pineal gland, via its hormone melatonin, has been shown to play a central role in the regulation of circadian rhythms as well as sleep\wake cycles. Furthermore, the synthesis of melatonin, which is increased in response to darkness, can be blunted and even blocked by exposure to light at night. As individuals age, there is the onset of disrupted sleep and nighttime exposure to light, leads to a significant suppression in the nocturnal levels of melatonin after age 60. Over the last two decades, considerable evidence has accumulated demonstrating that the pineal gland, via its hormone melatonin, possesses significant oncostatic activity, particularly for breast cancer. Our studies have demonstrated that melatonin is able to significantly suppress the transcription of the ER gene, and that it modulates the expression of estrogen-regulated growth-stimulatory factors, oncogenes, and proteins (TGF α , *c-fos*, pS2 and PgR), as well as increasing the expression of the potent growth-inhibitor TGF β , through the activation of a membrane associated G-protein-coupled receptor, the Mel1a (MT1) receptor. Therefore, given that melatonin levels diminish significantly by the 5th and 6th decades of life as the incidence of breast cancer increases, we hypothesize that the age related decline in pineal melatonin production leads to an enhancement of breast cancer development and growth in older women. Given that there are no well designed or tested models of aging and breast cancer the purpose of these studies is to define the age-related changes in melatonin and melatonin receptors, and thus, sensitivity to melatonin in response to age in the female Buffalo rat for the purpose of using the Buffalo rat transplantable mammary tumor model to test the above hypothesis. The scope of the research for the second year was to characterize age related changes in melatonin production and responsivity to melatonin in female Buffalo rats, and to define the growth of tumors in young vs. middle age vs. old female rats.

BODY:

Our studies have demonstrated that Buffalo rats are quite sensitive to the oncostatic effects of melatonin and to changes in photoperiod. However, the endogenous circadian rhythm of melatonin has not been fully characterized in this rat model. Given that responsiveness to exogenous melatonin is associated with endogenous melatonin synthesis and that the endogenous melatonin rhythm apparently changes with the onset of old age, it is essential that we fully characterize the differences in the circadian melatonin profiles in young, middle aged and old rats. To accomplish these goals we proposed the following Specific Aims for years 1 and 2.

1. **To characterize age related changes in melatonin production and responsivity to melatonin in Buffalo rats by:**
 - a. Examining melatonin rhythms in young, middle aged, and old Buffalo rats.
 - b. To characterizing the expression of the melatonin (MT1) receptor in the
 - c. hypothalamus and uterus of young, middle aged, and old Buffalo female rats.
 - d. Measuring responsivity to melatonin in young, middle aged, and old rats.
2. **To characterize melatonin's effects on the growth of transplantable N-nitroso-N-methylurea (NMU)-induced mammary tumors in young, middle aged and old female Buffalo rats by:**
 - a. Comparing mammary tumor growth in young, middle aged and old rats.
 - b. Determining the effects of exogenous melatonin on mammary tumor growth in young, middle aged and old rats.
 - c. Analyzing various aspects of cellular and molecular mammary tumor biology in response to melatonin in young middle aged and old rats

To accomplish the studies proposed in this specific aim we proposed following Statement of Work: *Since we had to age our own rats, we have included data from the first year that we have now filled out with data from old rats (20 months of age).*

First Six Months our tasks, as outlined in the grant were:

- To purchase young, middle aged and old Buffalo rats, and let them adjust to long day photoperiod (12L:12D), then collect serum and measure melatonin serum levels. These studies will define the differences in melatonin levels in young, middle aged and old rats that will serve as the baseline for future studies.

First Year:

- Determine the differences in serum melatonin levels in young, middle aged and old Buffalo rats.
- Characterize differences in melatonin receptor (MT1) expression in melatonin-responsive tissues (uteri) in young, middle aged and old Buffalo rats.

Second Year:

- Define which animals young, middle aged or old, are more responsive to exogenous melatonin by measuring ER, TGF β and RAR α and β expression in hypothalami and uteri of melatonin-treated rats.
- Determine the growth characteristics of tissue-isolated transplanted NMU mammary tumors in young, middle aged and old Buffalo rats, and define the molecular and cellular characteristics of these tumors in the different age groups.

Third Year:

- Compare the growth-inhibitory response of tissue-isolated transplanted NMU mammary tumors in young, middle aged and old rats to exogenous melatonin.
- To analyze the expression of various genes known to be melatonin-regulated and involved in tumor growth in tumors from young, middle aged and old rats in response to melatonin treatment.

For these studies we purchased female Buffalo rats, BUF (BUF/Ner) (National Cancer Institute) from Charles River Laboratories (Kingston, NY) at 4 weeks of age and maintained in environmentally controlled rooms in facilities (Tulane Vivarium). After 4 weeks in long day photoperiod (12L: 12D) two groups of Buffalo rats (10 rats in each group), at 2 month, 15 months of age, *and now 20 months of age*, corresponding to young, middle aged (adult), *and old* rats, respectively, were exsanguinated and truncal blood collected during the light phase (at 0900 and 1600 h), the dark phase (1800, 2000, 2300, 2400, 0100, 0200 and 0400 h) and then again at 0900 h. During the dark phase, blood samples were collected under a dim red light (Kodak Safelight) to avoid light-induced suppression of melatonin production (1). Melatonin levels were measured in the serum over a 24 h period using an ultrasensitive RIA for melatonin (2). We have used this assay previously for the measurement of melatonin levels in Buffalo rats (3). Data from these studies were analyzed by ANOVA simultaneously accounting for sources of variation principally conceived as treatments and time, with repeated measures where indicated.

One problem developed with this project that we had not anticipated. The supplier of the Buffalo rats, Harlan Sprague-Dawley, no longer maintains aged (20 – 30 month old) rats. Therefore, we have had to purchase these animals and have begun to age them. *As some of these rats reached old age (20 months or greater) in the last 6 months, we have finished the tumor growth studies, but are still in the process of concluding the studies of the molecular aspects of melatonin treatment.* We do anticipate that we will be able to complete the entire project (young, adult and aged rats) close to the proposed deadline, we should have our final data on the expression of ER α , MT1, RAR α in tumors in response to melatonin completed by the end of the summer.

Serum levels of melatonin: Our data as shown in Figures 1 and 2 demonstrate that in female Buffalo rats nocturnal serum melatonin levels diminish significantly from young rats (8 weeks of age) to adult rats (15 months of age) and *even to a greater extent in old (20 months of age) rats.* Figure 1 shows the diurnal rhythm of serum melatonin in young, middle age and old rats. As shown in this figure, a significant difference in the timing of the onset or offset of melatonin serum levels is evident between young and adult rats. With lights off at 1800 h (6:00 p.m.) and on at 0600 h (6:00 a.m.) melatonin levels in young rats began to rise between 1800 h and diminish, back to day time values, by 0500 h. In adult rats the onset of melatonin levels during the dark phase of the light:dark cycle was delayed to approximately 2000 h and returned back to daytime values by 0400 h. Thus, adult rats showed at 2-3 h reduction in the plateau of melatonin production. This decrease in the length of the plateau of melatonin was also accompanied by a significant ($p < 0.05$) decrease (29%) in the peak value of serum melatonin as shown in Figure 2. In young rats the mean peak serum levels of melatonin was 123 pg/ml of serum, while in adult rats mean peak serum levels of melatonin was 88 pg/ml. *In old rats (20 mo.) the onset of melatonin levels during the dark phase of the light: dark cycle was delayed to approximately 2250 h resulting in a 3-5 h reduction in the length of the melatonin plateau compared to young animals. As well the old female rats showed a highly significant diminution of peak serum melatonin levels from 123 pg/ml of serum in the young to 30 pg/ml of serum.*

Figure 1. Changes in female Buffalo rat melatonin rhythm with increasing age. Serum melatonin levels of 10 young (2 months of age), 10 adult (15 months of age) and 10 old (20 months of age) female Buffalo rats at time points of 0900 and 1600 h (light phase) and 1800, 2000, 2300, 2400, 0100, 0200 and 0400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 before being killed. The curve obtained for both ages is roughly sinusoidal, with low levels during the daytime and elevated levels at nighttime 

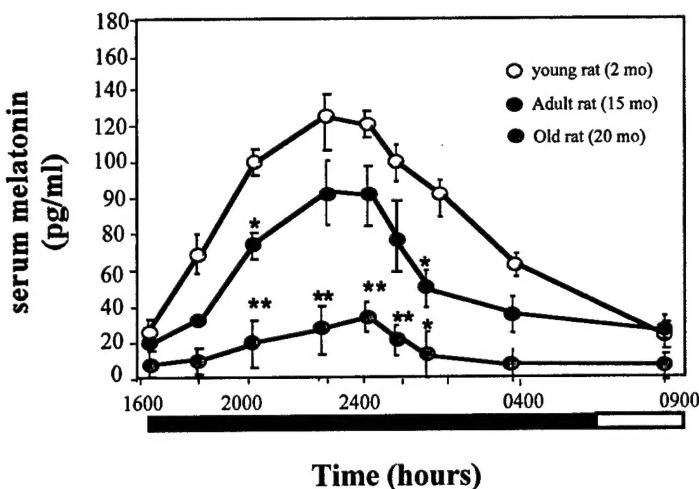
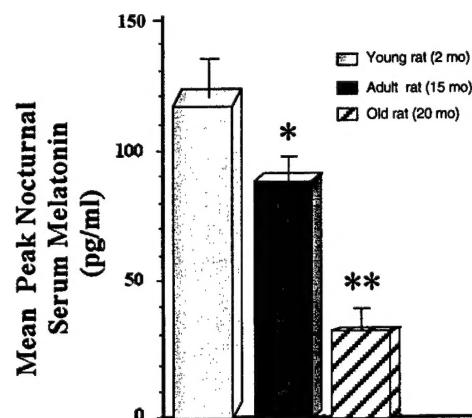


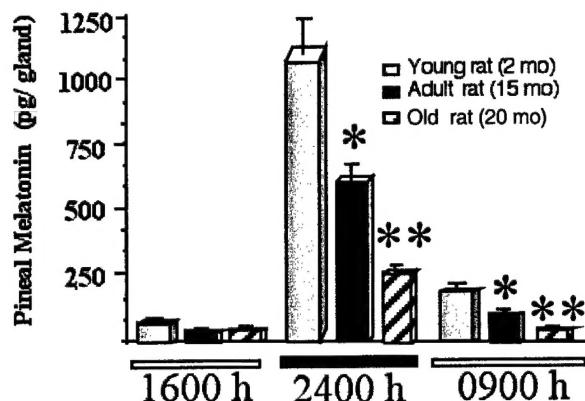
Figure 2. Peak daytime and nighttime serum levels of melatonin in adult and young female Buffalo rats. Serum melatonin levels of 10 young (2 months of age), 10 adult (15 months of age) and 10 old (20 months of age) female Buffalo rats at 2400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 before being killed under a red-light. * p<0.05 vs. young rats.



Pineal melatonin levels: In addition to examining changes in serum melatonin levels in young and adult Buffalo rats, we also examined pineal levels of melatonin in these same animals. Again, animals were kept in long day photoperiod on a 12 light: 12 dark cycle with lights off at 1800 h (6:00 p.m.) and on at 0600 h (6:00 a.m.). After 4 weeks in long day photoperiod (12L: 12D) three groups of Buffalo rats (10 rats in each group), at 2 month, 15 months of age and 20 months of age, corresponding to young, adult and old rats, respectively, were exsanguinated and during the light phase (at 0900 h) and the dark phase (2400 h). During the dark phase (2400 h), pineal samples were collected under a dim red light (Kodak Safelight) to avoid light-induced suppression of melatonin, frozen on solid CO₂. Pineal glands were then stored frozen at -20° C until melatonin assays were performed 3-10 days later. Melatonin levels were measured in the serum over a 24 h period using an ultrasensitive RIA for melatonin as described above. Figure 3 shows daytime and nighttime pineal melatonin levels in young (2 months), middle aged (15 months of age) and old (20 months of age) female rats. Middle aged adult rats in this study showed a significant (p<0.01) diminution of nighttime pineal melatonin levels compared to young rats, while old age rats showed a highly significant diminution of pineal melatonin compared to both young and middle age rats. At this time, the melatonin content of the

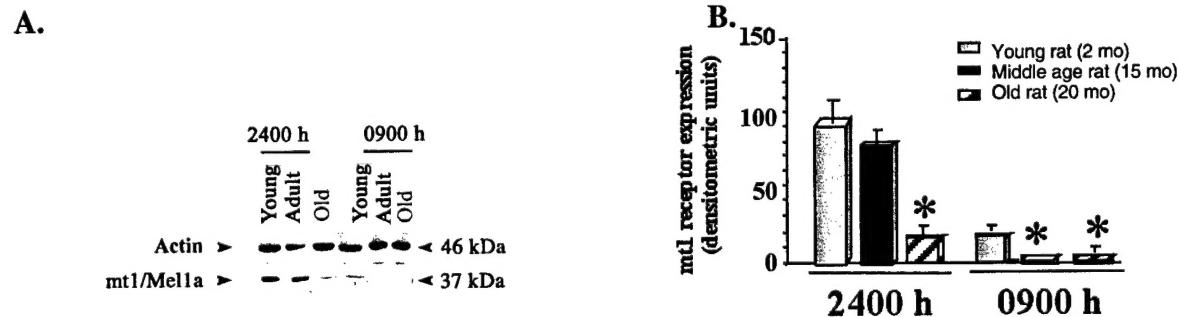
pineal glands of the young rats exceeded daytime levels by 13-fold, whereas in the middle aged, adult, rats only a 7-fold increase in nocturnal levels of pineal melatonin were observed. *In the old rats only a 3-fold increase in nocturnal levels of pineal melatonin were observed.*

Figure 3. Pineal melatonin content in young and adult female Buffalo rats. Pineal melatonin levels of 7 young (2 months of age), 10 adult (15 months of age) and old (20 mo) female Buffalo rats collected at 2400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 and were exsanguinated under a dark-light. * p<0.05 vs. young rats.



Uterine expression of the melatonin MT1 receptor: It has been well demonstrated that melatonin can modulate uterine function. For example, female hamsters placed in long day photoperiods will have fully developed uteri and administration of melatonin, late in the afternoon, will induce the involution of the uterus. As shown in Figure 4, female Buffalo rats express quantifiable levels of the melatonin MT1 receptor. This figure also shows that uterine levels of the MT1 receptor are diminished by 41% in middle age adult rats and by 80% in old rats (20 months of age) compared to young rats.

Figure 4. Uterine melatonin MT1 receptor expression in young and adult female Buffalo rats. (A) Melatonin MT1 receptor protein levels were measured by Western blot analysis, using the 563 anti-MT1 antibody, provided by Dr. Rolf Jockers (Paris, France), from total cellular protein isolated from the uteri of 10 young (2 months of age), 10 adult (15 months of age) and 4 old (20 months of age) female Buffalo rats at 2400 h (dark phase) and 0900 h (light phase). Animals were maintained in a light:dark cycle of 12:12 and a red-light was used when animals were exsanguinated during the dark phase. (B) Densitometric analysis of MT1 Westerns from 4-10 different animals in each group. * p<0.05 vs. young rats.



Tumor Growth as a Function of Age and Melatonin Treatment: It has been well documented that melatonin can significantly suppress the growth of N-nitroso-N-methylurea (NMU)-induced mammary tumors in rat's (1). Using a tissue-isolated transplantable model tumor that we have previously employed (3), we examined the growth of NMU-mammary tumor transplants in response to age or melatonin as a function of host age. Briefly, a 3-mm cube of tumor was sutured to the tip of the vascular stalk formed from the superficial epigastric artery and vein. The tumor implant and vascular stalk were enclosed within a parafilm envelope and placed in the inguinal fossa, and the skin incision closed. Vascularization of the implant is limited to new vessel connections with the epigastric artery and vein: subsequent tumor growth is s.c. When tumors reached sufficient size for measurement, rats were subjected to light CO₂ narcosis and tumor dimensions were measured through the skin with vernier calipers and were converted to tumor weights as described previously (3). Growth rates (g/day) were generated by linear regression from the estimated tumor weights for the treated and control animals. The final tumor weight was determined by weighing at the end of the experiment. The average tumor after 25 days of growth, as shown in Figure 5, was estimated at 9.5 g in the young rats vs. 9.4 g in the middle-aged rats and 16.9 g in old rats. Overall, tissue-isolated tumors transplanted in to old rats grew significantly faster and were larger (1.9-fold or 80% larger) than tumor sections (from the same tumors placed into young or middle aged rats. Tumor weight in response to exogenous melatonin treatment resulted in an average tumor weight at the end of the study of 4.9 g in young rats, 3.2 g in middle aged rats and 11.3 g in old rats. This corresponds to a 48% decrease in tumor weight in young rats treated with melatonin, a 66% decrease in tumor weight in middle aged rats in response to melatonin, but only a 33% decrease in tumor weight in old rats following daily melatonin administrations (500 µg/day). Thus, in old rats there is a highly significant 2-fold reduction in response to the growth-suppressive actions of melatonin, as compared to middle aged rats. Mammary tumors from old rats treated with melatonin were 2.3- and 3.5-fold larger than melatonin-treated tumors from their young and middle aged counterparts, respectively. Thus, a highly significant enhancement of mammary tumor growth was seen in old rats, as compared to young and middle-aged rats, and as well, a highly significant suppression in melatonin-responsiveness was noted in the old rats as compared to young and middle-aged rats.

Cellular and Molecular Analysis of Melatonin-Modulated Receptors and Proteins. Previous studies by our laboratory has demonstrated that treatment of human breast tumor cell lines with melatonin results in the down-regulation in the protein levels of ER α and the potent autocrine and paracrine growth factor, Transforming Growth Factor- alpha (TGF α) [4]. Tumor progression is often associated with a loss of estrogen responsiveness and an enhanced response to growth factors as their primary mitogens. As well, for a tumor to be responsive to the growth-suppressive actions of melatonin, the cells must express reasonable levels of the MT1 or MT2 receptor. As we have reported that breast tumor cells only express the MT1 receptor we have examined the expression of this receptor in our tumors. As shown in Figure 6 we have examined the protein expression of ER α , MT1 and TGF α in our tissue-isolated NMU-induced mammary tumors taken from young, middle aged and old rats. As we have just recently terminated the tumor-growth studies, we have not yet completed this last section of our project. However, we have completed 2 independent runs of our Western blot analyses. Figure 6 clearly demonstrates that ER α and MT1 levels are

decreased in tumors growing in old rats as compared to young and middle aged rats, while TGF α expression appears to be greatly enhanced in tumors growth in the older rats. Since we have only completed an analysis of 2 tumors each from young, middle aged and old rats no statistical analysis is able available. As we complete these cell and molecular analyses of tumors statistical significance will be assigned to changes in each group.

Figure 5 Effects of age and melatonin on the growth of tissue-isolated NMU-induced mammary tumors in Buffalo rats. (A) Tumor growth in Young rats (2 mo.), (B) tumor growth in Middle Aged rats (12 mo.), (C) tumor growth in Old rats (20 mo.) in response to diluent (0.1 ml of ethanolic saline) or melatonin (500 μ g/0.1 ml) injections administered every day in the late afternoon (4:00-6:00 p.m.) to rats provided with a semipurified diet containing 5% corn oil *ad libitum*. Regression analysis and tests for parallelism indicate that melatonin treatment significantly (* $P<0.05$) decreased the tumor growth rate in both the young and middle aged animals. (D) The average estimated tumor size after 25 days of growth between controls and melatonin treated rats in young, middle aged, and old rats.

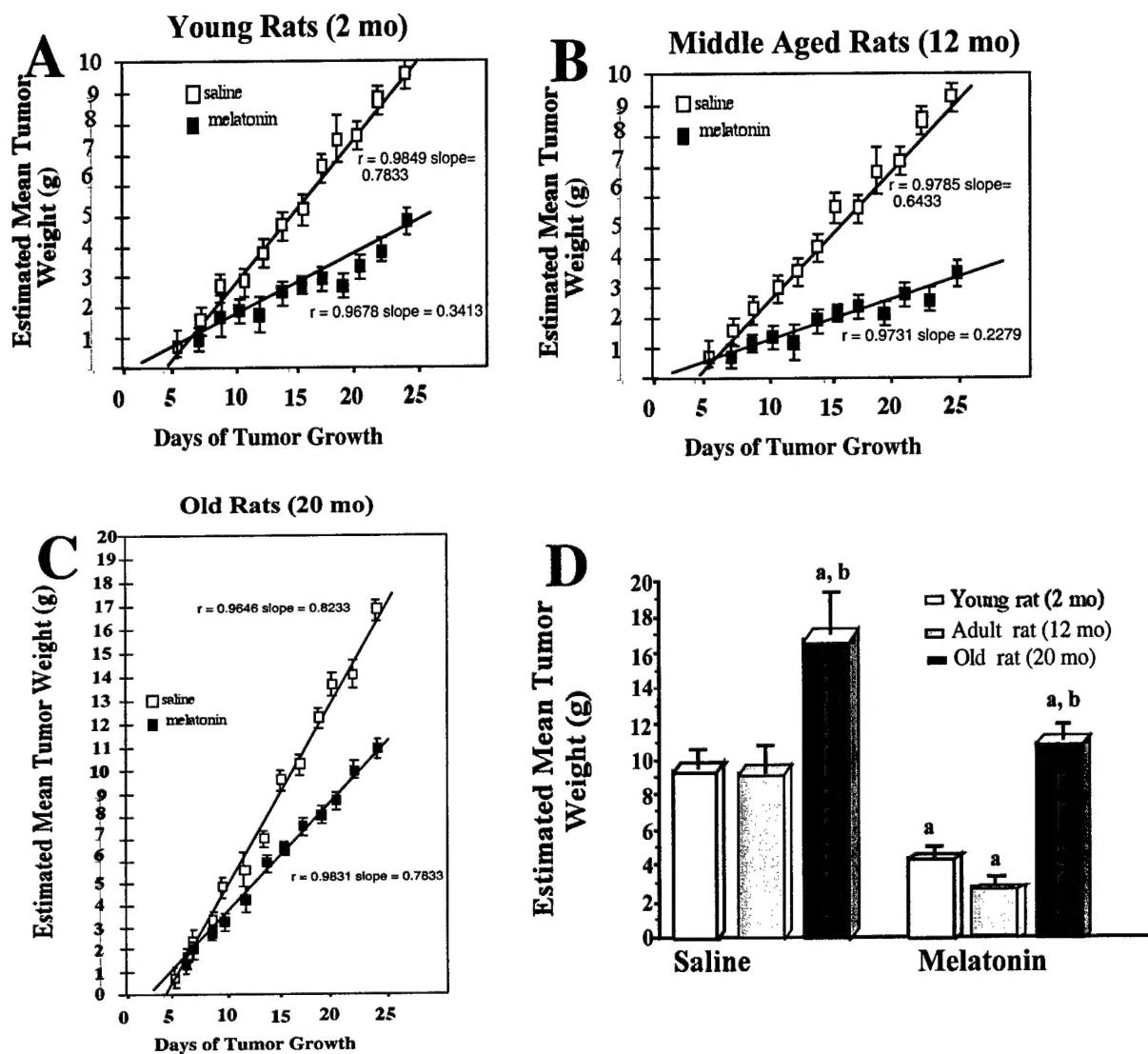
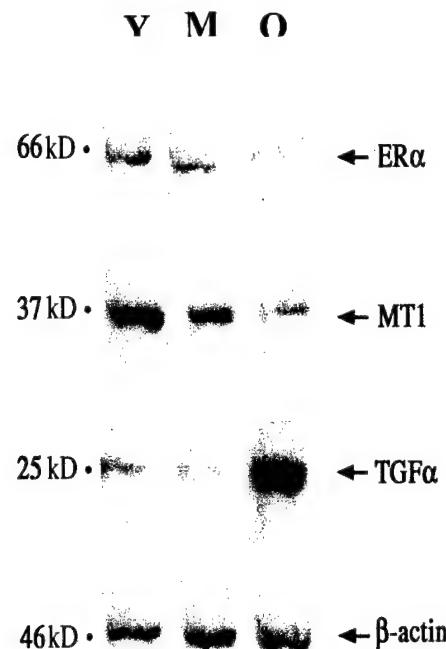


Figure 6. Western blot analysis of ER α , MT1 and TGF α expression in tissue-isolated tumors from young, middle aged and old Buffalo rats. After 25 days of growth, tumors were isolated and total cellular protein isolated using appropriate protease inhibitors. Fifty micrograms of total cellular protein from each tumor was fractionated on a 12% SDS-polyacrylamide gel, transferred to Hybond membrane, and incubated with monoclonal antibodies against, ER α , MT1 and TGF α . This figure is a radiograph of one run of Western blot analysis for each of the proteins of interest and β -actin, which is used to control for protein loading.



KEY RESEARCH ACCOMPLISHMENTS:

- The onset and offset of the melatonin production was significantly delayed and retracted, respectively, in old rats compared to young and adult rats, so that the phase of melatonin production was significantly delayed in old rats compared to middle aged and young rats, and in middle aged rats compared to young rats.
- The peak nocturnal serum melatonin level was significantly blunted in old buffalo, as compared to young and adult rats, to the point that it was in some old rats it was not significantly greater than the day values seen in young rats.
- The nocturnal production of pineal melatonin was significantly blunted (by 2.6- and 4.5-fold) in the pineal glands of old (20 months) Buffalo rats compared to adult (15 month) and young (2 months) rats, respectively.

- The uterine levels of MT1 receptor were significantly diminished by 79% in uteri from old rats as compared to young and even middle-aged rats.
- The growth rate of transplantable mammary tumors in middle age rats was not significantly altered compared to young rats, while growth-suppression by melatonin was marginally reduced in middle age rats compared to young rats. In old rats the growth of the tissue-isolated tumors were significantly enhanced as compared to that in young and middle aged rats. Tumor response to the growth suppressive actions of melatonin was significantly blunted in the old rats as compared to the young and middle aged rats.
- The expression of ER α , the receptor for the mitogenic estrogen-response pathway, is considerably diminished in mammary tumors from old vs. middle age and young rats. A similar decrease in MT1 melatonin receptor expression is seen in tumors grown in the aged rats as compared to the young and middle aged rats. Finally, the expression of TGF α , a potent mitogenic growth factor was greatly enhanced in tumors isolated from the aged rats but not in the young and middle aged rats.

REPORTABLE OUTCOMES:

- Army/DoD report
- Abstract at the 2002 Era of Hope meeting in Orlando, FL

CONCLUSIONS:

The major question addressed in this project is whether melatonin levels and sensitivity are diminished with advancing age and if these changes make aged rats more susceptible to mammary tumor development. In our second phase of this project we have demonstrated that there is a significant 4-fold decline in peak serum melatonin levels in old female Buffalo rats compared to young rats. In addition, the period of melatonin release is significantly shortened in old as compared to middle age and young rats. These data correlate with the decline in pineal melatonin production in the old rats as compared to middle age and young rats. As well, we have demonstrated that in old rats, that the levels of the MT1 receptor is dramatically diminished (by almost 80%) in uterine tissues during the light phase of the light/dark cycle compared to both middle age and young rats. It is important to note that MT1 receptor levels are as well reduced in middle aged rats compared to young rats. Thus, if melatonin does possess antitumor activity with regards to breast cancer, we would expect old rats to be more susceptible to the formation of mammary tumors, based on their reduced levels of endogenous melatonin.

Our data with the tissue-isolated transplantable mammary tumors in young vs. middle age rats indicates that the moderate reduction in melatonin levels in middle age rats does not affect tumor growth overall compared to tumors grown in young rats, but does appear to make them slightly more sensitive to the growth-suppressive actions of exogenous melatonin. The studies with tissue-isolated transplantable mammary tumors in

old rats are very compelling. In the tumors transplanted into the old rats, tumors grew at a significantly faster rate and as expected tumor size after 25 days of growth was almost 2-fold greater than in middle aged and young rats. In tumors isolated from the old rats, we found that ER α and MT1 melatonin receptor levels were greatly reduced, while the expression of the potent mitogen TGF α was greatly enhanced. These data suggest that in response to the lack of estrogen and melatonin in the old rats, the tumors appear to down-regulate the receptors for these hormones, effectively, ablating the mitogenic estrogen-pathway and further suppressing the growth-inhibitory melatonin-signaling pathway. As well, the enhanced expression of TGF α suggests, the tumors have turned on their growth-factor pathways as their primary mitogenic pathway. Although melatonin treatment of tumors in old rats induced a significant (33%) inhibition of tumor growth compared to diluent treated controls in old rats; this growth-suppressive action of melatonin was significantly less than that seen in either middle aged or young rats. These data suggest, that with enhanced age, the decline in melatonin production may lead to a down-regulation of MT1 melatonin receptors, even in transplanted tumors, reducing the tumors sensitivity to the growth-suppressive actions of melatonin, and allowing greater tumor growth. Finally, these data demonstrate that we have developed a reasonable model for the study of aging and breast cancer.

REFERENCES:

1. Blask, D.E., Pelletier, D.B., Hill, S.M., Orstead, K.M., and Massa, J.S. Pineal melatonin inhibition of tumor promotion in the N-nitroso-N-methylurea model of mammary carcinogenesis: Potential involvement of antiestrogenic mechanisms *in vivo*. *J. Cancer Res. Clin. Oncol.*, 117:526, 1991.
2. Vaughn, G.M. New radioimmunoassay for the measurement of melatonin. *J. Pineal Res.*, 15:88, 1993.
3. Blask, D.E., Sauer, L.A., Dauchy, R.T., Holowachuck, E.W., and Fuhoff, M.S. Melatonin regulation of tumor growth and the role of fatty acid uptake and metabolism. *Neuroendocrinol. Lett.*, 18:59, 1997.
4. Molis, T., Spriggs, L., Jupiter, Y., and Hill, S.M. Melatonin modulation of estrogen-regulated proteins, growth factors, and proto-oncogenes in human breast cancer. *J. Pineal Research*, 18:93-103, 1995.

APPENDICIES:

Abstract for Era of Hope 2002

Final Report DAMD17-00-1-0473

Project Dates 6/1/00 –5/30/03

Annual Reports submitted:

6/01

6/02

poster and platform presentation 7/03 Era of Hope Meeting, Orlando, FL

Presonnel receiving pay from this research effort:

Steven M. Hill, Ph.D. – Principal Investigator

Lin Yuan, M.D. (00-02)

Qi Cheng, M.D. (02-03)

Appendix
(Abstract, Era of Hope 2002)

**AGE RELATED DECLINE IN SERUM AND PINEAL MELATONIN LEVELS AND
UTERINE MT1 MELATONIN RECEPTOR LEVELS IN YOUNG AND MIDDLE-AGED
FEMALE BUFFALO RATS.**

Steven M. Hill, Ph.D. and Qi Chang, M. D.

Department of Structural and Cellular Biology, Tulane Cancer Center, Tulane University Health
Science Center, 1430 Tulane Avenue, New Orleans, LA 70112

E-mail:smhill@tulane.edu

Over the last two decades, considerable evidence has accumulated demonstrating that the pineal gland, via its hormone melatonin, possesses significant oncostatic activity, particularly for breast cancer. Our studies have demonstrated that melatonin is able to significantly suppress the transcription of the ER gene, and that it modulates the expression of estrogen-regulated growth-stimulatory factors (TGF α , TGF β , *c-fos*, pS2 and PgR) through the activation of a membrane associated G-protein-coupled receptor, the MT1 melatonin receptor. As individuals age, there is the onset of disrupted sleep and nighttime exposure to light, leading to a significant suppression in the nocturnal levels of melatonin after age 60. Therefore, given that melatonin levels diminish significantly by the 5th and 6th decades of life as the incidence of breast cancer increases, we hypothesize that the age related decline in pineal melatonin production leads to an enhancement of breast cancer development and growth in older women. Given that there are no well designed or tested models of aging and breast cancer the purpose of these studies is to define the age-related changes in melatonin and melatonin receptors, and thus, the response of transplanted carcinogen-induced mammary tumors in the young, middle-aged and old female Buffalo rats.

To begin to define the melatonin profile in young, middle-aged and old female Buffalo rats, serum and pineal melatonin levels were measured in young (8 months of age) and middle-aged (15 months of age) rats. Serum melatonin was measured at 0900 and 1600 h (light phase) and 1800, 2200, 2300, 2400, 0100, 0200 and 0400 h (dark phase) of 10 young and 10 middle-aged female rats. Our data demonstrate that in female Buffalo rats nocturnal serum melatonin levels diminish significantly from young to adult rats, with a significant difference in the timing of the onset of melatonin serum levels evident between young and adult rats. In adult rats the onset of the evening melatonin rise was delayed by approximately 2-3 h. This delay in the onset of the melatonin plateau was also accompanied by a significant ($p < 0.05$) decrease (29%) in the peak value of serum melatonin (mean peak melatonin serum level of 123 pg/ml and 88 pg/ml of serum in young and adult rats, respectively) in middle-aged rats. The level of pineal melatonin was also examined in these same animals. Middle aged adult rats in this study showed a significant ($p < 0.01$) diminution of nighttime pineal melatonin levels compared to young rats. The nighttime melatonin content of the pineal glands of the young rats exceeded daytime levels by 13-fold, whereas in the middle aged rats only a 7-fold increase in nocturnal level of pineal melatonin was observed.

It has been well documented that melatonin can modulate uterine function in hamsters. Our studies demonstrate that the uteri of female Buffalo rats express quantifiable levels of the MT1 melatonin receptor and that the levels of this receptor are diminished by 41% in adult rats compared to young rats. Currently, studies are underway comparing the tumor take and growth of N-nitro-N-methylurea (NMU)-induced tumors transplanted into young and middle-aged Buffalo female rats